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14. ABSTRACT
The primary objective of this grant was to determine the relapse free survival benefit with locally advanced and Stage IV HER2+ breast cancer patients vaccinated with a HER2 ICD peptide-based vaccine while receiving maintenance trastuzumab. The scope of work includes a Phase II single arm study of a HER2 ICD peptide-based vaccine given concurrently with trastuzumab. Thirty six subjects have been enrolled to this study. All adverse events reported for are of low grade and there is no adverse cardiac toxicity with the combination of vaccine and trastuzumab. Patients have developed significant T-cell immune responses to the vaccine as well as epitope spreading indicating a Type I immune response is being elicited. Preliminary interim analysis on the first 25 subjects, suggests clinical benefit with a 2-year RFS estimate of 63% and the one-year RFS estimate is 82%. Overall survival evaluation in the first 25 subjects is due November 2011. Relapse free survival evaluation is ongoing as this follow-up will be 4 years after last vaccine for the last enrolled subject.

15. SUBJECT TERMS
Phase II, Clinical Trial, HER2, trastuzumab

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INTRODUCTION

This study is a Phase II single arm clinical trial of a HER2 ICD peptide based vaccine given concurrently with trastuzumab. Enrolled patients included either: (1) patients with locally advanced HER2-positive breast cancer (Stage IIIB and IIIC) who were in complete remission and within 1 year of diagnosis and initiating treatment with chemotherapy and trastuzumab or (2) Stage IV HER2-postive breast cancer patients who were in their first complete remission and defined as NED (no evidence of disease) or had stable bone only disease and were within 6 months of starting maintenance trastuzumab. The primary objective is to estimate relapse free survival compared to a historical control of patients treated with chemotherapy and trastuzumab (44% at 4 years). We hypothesize that the relapse free survival rate at 4 years with vaccination, if successful, would be 58%. Fifty-two patients will provide 92% power to detect a statistically significant increased survival rate compared to the fixed historical rate of 44% at the one-sided significance level of p=0.05.

Secondary objectives include the assessment of the toxicity of the combined approach as well as the immunogenicity of HER2 ICD peptide vaccination. If there is evidence to suggest that the true rate of Grade IV toxicity exceeds 5% or the true rate of Grade III-IV toxicity exceeds 10% then the trial will be stopped for safety concerns. Immunogenicity of the approach will be evaluated as the ability of the vaccine to elicit HER2 ICD specific T cell immunity, to elicit epitope spreading, and to stimulate both a CD4+ and CD8+ immune response. Immune response and epitope spreading will then be modeled as time-dependent covariates in Cox proportional hazards regression models for overall survival (OS) to assess the correlation of each of these outcomes with relapse.

BODY

Task 1*: To assess the potential clinical impact of the administration of a HER2 ICD peptide-based vaccine to Stage IV breast cancer patients receiving concurrent trastuzumab monotherapy*

a. Construct and vial the HER2 ICD peptide vaccine. This task has been completed. The vaccine product (lot 6002) continues to be monitored at specific intervals for product stability. A Stability Study Log for lot 6002 is maintained. The study log lists the testing dates and provides a summary table to record data for each time point tested. All reserved stability vials are stored under the same conditions as the final product, -20 ± 2°C. At each stability time point reserved vials are removed from storage and visually inspected for appearance. MALDI-TOF mass spectrometry and High Performance Liquid Chromatography (HPLC) are used to confirm the stability.

Testing is performed regularly; Table 1 provides a list of test times and outcomes. In the last three tests we have observed dimerization of this vaccine. We have developed an ELISPOT assay to assess the ability of our stored vaccine to stimulate peptide specific T cell immune responses. In the assay, we use four concentrations of ICD vaccine and peptide mixture (0.1, 1, 10 and 20ug/ml) respectively to stimulate T cell responses in donors. According to the data from 10 day ELISPOT assay, the stored ICD peptide based vaccine exhibited similar ability to elicit peptide specific T cell responses *in vitro* as compared to recently constructed and purified peptides. This assay serves as a functional validation of the continuing immunogenicity of the stored vaccine.

Table 1: Product Stability Testing Results

Testing Days	Stability Conditions Met?	Dimerization D	imeriz ation Outcomes
90	YES	None	Not applicable
180	YES	None	Not applicable
270	YES	None	Not applicable
360	YES	None	Not applicable
540	YES	None	Not applicable
720	YES	9%	Still able to elicit peptide specific T cell responses as compared to recently constructed and purified peptides.
1080	YES	14.8%	Still able to elicit peptide specific T cell responses as compared to recently constructed and purified peptides.

Testing Days	Stability Conditions Met?	Dimerization D	imeriz ation Outcomes
1440	YES	18.8%	Assay is on-going at this time
1825	YES	19.9%	Assay is on-going at this time

b. Enroll and treat patients. This study was originally submitted on May 2004. This study was officially approved by the US Army Medical Research and Materiel Command (USAMRMC) Human Subjects Research Review Board (HSRRB) on June 1, 2006. To date we have enrolled a total of 36 subjects.

When this study was initially designed, the standard convention for use of trastuzumab was in patients with HER2 positive stage IV breast cancer (only FDA approved indication) and occasionally in HER2 positive stage III patients enrolled in trastuzumab protocols. Data from the 2 large NCI clinical trials (NSABP: B-31 and NCCTG: N9831) which demonstrated that adjuvant trastuzumab improved survival outcomes in HER2 positive early stage breast cancer were first reported via NCI press release in April, 2005 and then presented at ASCO in May 2005. These findings had immediate impact on the current clinical practice; and by July 2005, based on the NCI trials data, adjuvant trastuzumab was being offered to patients with early-stage HER2 positive breast cancer. Soon after, in October 2005, data from the Breast International Group (HERA Trial) showed similar trastuzumab-related survival benefits to that seen in the NCI trials; and the addition of trastuzumab to adjuvant systemic chemotherapy in early stage HER2 positive breast cancer became the new standard of care.

When the protocol was originally submitted to the DOD for IRB approval in May 2004, trastuzumab therapy was only approved for and given to HER2 positive Stage IV breast cancer patients. Therefore, our original targeted study population was defined as trastuzumab-naïve HER2 positive Stage IV patients in first relapse who would be receiving trastuzumab in the first-line salvage setting followed by HER2 vaccination.

However, the study did not receive DOD IRB approval until June 2006 and we were not able to enroll the first study subject until December 2006. So in the 2.5 year period between DOD submission, DOD IRB approval and subsequent initiation of study enrollment, the standard of care had changed and the majority of early stage (stage II and III) HER2 positive patients were receiving trastuzumab in the adjuvant setting as part of their standard therapy. So patients previously diagnosed with early stage HER2 positive breast cancer and now presenting with their first episode of relapse breast cancer (Stage IV disease) had more than likely received trastuzumab as part of their earlier therapy and were no longer trastuzumab-naïve nor eligible for enrollment to this study. It would soon become evident that our original targeted study population was rapidly shrinking given that only those patients presenting with de novo stage IV disease (which comprises only about 6%-10% of all stage IV breast cancer cases) would be trastuzumab-naïve and eligible for study. Eventually, these findings prompted changes to the protocol including changes to the targeted population and primary endpoint.

For this reason we opened the trial up to both Stage IIIB and IIIC patients to boost accrual to this study. The literature demonstrates that both Stage IIIB and IIIC receive similar treatment of neoadjuvant and adjuvant chemotherapy in combination with trastuzumab for up to 12 months. In addition the overall survival (OS) and relapse free survival (RFS) is similar between the two groups and Stage IV patients.

However, adding Stage IIIB and Stage IIIC to the study it did result in a change to the study protocol. We consulted our biostatistician, Dr. Katherine Moore (Grants Manager, CDMRP) and Ms. Young on April 8, 2008 to agree on proposed changes that would not significantly altering study design be still allow us to meet our primary endpoint of clinical response.

The result was that the primary endpoint to evaluate RFS and OS in locally advanced (Stage IIIB and IIIC) and Stage IV breast cancer as one group collectively. However, at the same time we wanted to keep the sample size the same, as well as conduct the scheduled interim analysis after 25 patients to determine if we should continue with enrollment. Therefore, the primary objective was modified to:

> "To estimate the RFS in patients with HER2+ locally advanced and Stage IV breast cancer vaccinated with a HER2 ICD peptide-based vaccine."

To date 22 subjects have completed the vaccines and follow-up procedures. We have 3 subjects that have complete the vaccine series and are still undergoing follow-up procedures. We have had 9 subjects withdrawal from study early (Table 2).

Table 2: Withdrawal details

Subject ID	Reason for Withdrawal
12007	Progression of disease after completion of 6 vaccines
12009	Off study at request of study physician
12013	Progression of disease after completion of 1 vaccine
12024	Progression of disease after completion of 2 vaccines
12027	Progression of disease after completion of 6 vaccines
12029	Off study due to starting a concomitant medication
12031	Off study due to adverse event of "rash"; study physician made the decision
12033	Off study due to adverse event of "pain in back"
12035	Progression of disease; brain metastases after completion of 1 vaccine

c. Interim statistical analysis after 25 patients have been followed for 1 year. In May 2010, thirty-two of 52 patients had been enrolled in this phase II study designed to prospectively evaluate RFS and OS in HER2+ locally advanced and metastatic breast cancer patients. Historical data would suggest 4-year (after diagnosis) relapse-free survival (RFS) rates for this patient cohort to be 44%. Our trial was powered to provide a significantly increased survival rate compared to the fixed historical rate of 44%. Thus, our benchmark to indicate vaccine efficacy would be a 4-year RFS of 58% or greater in our vaccinated cohort. A planned interim analysis of relapse-free survival in the first 25 patients who have completed vaccination was incorporated as part of the original study design. This formal interim analysis is scheduled for November 2011 at which time the first 25 patients will have completed at least two years of follow-up post vaccination. Additionally, a preliminary interim analysis was conducted in May 2010 on the first 25 enrolled patients with the 25th patient being at least 1 year out from the last vaccine. While, not all 25 vaccinated patients had completed follow-up to two years, there is sufficient preliminary evidence to suggest a 2-year RFS estimate of 63% and the one-year RFS estimate is 82%. Given the data thus far observed, and understanding that follow-up is limited at this point, treatment with HER2 vaccination appears to improve RFS.

d. Final analysis of response. The primary objective of this study is to evaluate RFS in HER2+ metastatic breast cancer. Relapse free survival was assessed 4 years after the last vaccine on the last subjects.

The study was originally designed to accrue 52 subjects. However, due to the unanticipated delays in the initial study approval and subsequent related difficulties in accrual of our original targeted study population (as described in Section Task 1.b. above) the funding for the study has ended. Thus, we recognize the on-going challenge of being able to achieve the initial target enrollment of 52 and we have decided to end accrual at 38 subjects after consulting with the study biostatistician, Dr. Ted Gooley, on November 4, 2010. Per Dr. Gooley, a sample size of 38 patients would still allow for adequate power and statistical significance to meet the primary objective. Specifically, 38 patients will provide 85% power to detect a statistically significantly increased survival rate compared to the fixed historical rate of 44% at the one-sided significance level of .05.

Table 3 provides a summary of the power and statistical significance if we were to accrue anywhere between 35 – 40 patients.

Table 3: Summary of statistical significance

Number of Subjects Accrued	Power Significance	(p-value)
35	82%	0.05
38	85%	0.05
40	87%	0.05

Thus, a total of 38 subjects will be enrolled and all subjects will be followed for RFS. We shall consider the proposed treatment to be potentially efficacious if the estimated 4-year RFS is 58% or greater. This estimate will be made when the minimum follow-up among all patients is 2 years.

Task 2*: To evaluate the safety of administering a HER2 ICD peptide-based vaccine to Stage IV breast cancer patients receiving trastuzumab monotherapy.*

a. Evaluate immediate toxicity associated with the vaccine. We use the NCI Common Toxicity Criteria (CTC) for Adverse Events Version 3.0 to grade toxicities. We pay particular attention to local reactions associated with the injection site and systemic reactions to include but not limited to fever, malaise, myalgia, nausea and headache. Table 4 summarizes the most common adverse events experienced during the study trial to date.

Table 4. Summary of Most Common Adverse Events

Adverse Events (AE)				
	All AE		Possibly, Probably, or Definitely Related	
Most Common Adverse Events	**n**	**% of all AE**	**n**	**% of All Related AE**
Injection site reaction	70	18	70	26
Fatigue	17	4	8	3
Hemoglobin	16	4	10	4
Hypokalemia	14	4	10	4
Lymphocytes	14	4	11	4
Leukocytes	14	4	11	4
Headache	10	3	7	3
Hypocalcemia	9	2	7	3
General rash	9	2	5	2
Renal – positive leukocytes	8	2	8	3
Nausea	8	2	4	1
Hypoalbunemia	8	2	8	3
Flu-like Syndrome	7	2	4	1
Diarrhea	7	2	3	1
Myalgias	7	2	5	2
Arthralgia	6	2	6	2
Neutrophils	6	2	5	2
	All AE		**Possibly, Probably, or Definitely Related**	
Adverse Event Grading	**n**	**%**	**n**	**%**
Grade 1	352	88	245	90
Grade 2	48	12	27	10
Grade 3	0	0	0	0
Grade 4	0	0	0	0
Grade 5	0	0	0	0

Please note that Table 4 records the most common adverse events regardless of severity. For example, a subject may have an injection site reaction at each of the three vaccines where another may not. Each one of these injection site reactions is recorded for that one subject.

b. Determine whether there is any cardiac toxicity associated with the co-administration of the HER2 ICD peptide based vaccine with trastuzumab. When subjects are enrolled we will closely monitor and document any abnormal cardiac events observed by us at clinic visits or reported to us by the subjects or physicians. All subjects have documentation of a MUGA/ECHO scan within 6 months for eligibility assessment and if that MUGA/ECHO scan is greater than 60 days old at time of eligibility we perform a MUGA/ECHO scan at their baseline visit. A follow-up MUGA/ECHO scan is performed again at 4 months post-vaccine. Table 5 compares ejection fractions at baseline and 4 Months Post-Last Vaccination.

Table 5: Baseline and 4 Month Post-Last Vaccine EF Evaluation

Subject # (n=23)	Pre-vaccine EF	4 months post-vaccine EF
12001	68%	60-65% (Echocardiogram)
12002	61%	65%
12003	65%	52%
12004	64%	Have not received follow-up documentation. Emailed physician for

Subject # (n=23)	Pre-vaccine EF	4 months post-vaccine EF
		copy of report, we did not get a response.
12005	59%	57.5%
12006	64%	61%
12007	60%	Subject's disease progressed prior to follow-up visit.
12008	66%	51-53%
12009	56%	45.8% [a]
12010	51-52%	Subject went off Herceptin before this visit. A MUGA was not performed by her oncologist
12011	57%	55%
12012	64%	69%
12013	69%	Off Study: Progressive Disease
12014	51%	51%
12015	66%	65%
12016	50%	46%
12017	55%	60%
12018	51%	57%
12019	68%	70.8%
12020	60%	60%: This was done as an ECHO by the subject's own physician
12021	55-65%	55-65%
12022	52%	56%
12023	51%	61%
12024	62%	Off Study: Progressive Disease
12025	57%	Not yet received
12026	59%	68%
12027	64%	64%; this was performed early
12028	66%	Last MUGA performed 12/14/2009; subject had been off Herceptin since March 2010. 12/14/2009 MUGA result: 68.10%
12029	60-65%	Off Study: Viral infection (high vial load)
12030	59%	Not yet due
12031	69%	Off Study: Developed rash after vaccine 1 and her physician encouraged her to stop participation
12032	57.3%	Not yet received; requested by Research Nurse
12033	65%	Off Study: Back pain after vaccine 1
12034	77%	Not yet due
12035	59.3%	Off Study: Disease progression after vaccine 1
12036	60%	Not yet due

[a] Primary oncologist is aware of EF drop. Off study per subject and last communication with her was March 25, 2008.

Two cardiac events have been observed (reported in the previous progress report), both reported during vaccination:

1. Grade 1 palpitations – Patient reports one episode of palpitations while watching TV and resolved spontaneously after about 5 minutes. No other related symptoms were reported. No other reports of palpitations have been reported since this one episode. Last episode of palpatations was one year ago while the subject was on chemotherapy.
2. Grade 2 hypotension – After vaccination 4, which included a large blood draw, patient felt presyncopal and she had to sit down. The medics were called and she had a BP of 90/50. She received IVF in transit, in ER and also when discharged home. Patient did not lose consciousness. Resolved by following study visit.

It should be noted that both of these events were for the same subject (ID#: 12010).

In conclusion, we did not find any significant cardiac toxicity between trastuzumab and HER2 ICD vaccine.

c. Evaluate for any potential toxicities due to the generation of an immune response to HER2. The toxicities we would expect to see for an autoimmune response to HER2 would include: (1) skin reactions such as rashes,

(2) gastrointestinal events such as severe diarrhea, (3) pulmonary events, (4) change in kidney function such as a change in creatinine or (5) cardiotoxicity. All of these toxicities are closely monitored, by a credentialed clinician such as a physician and/or physician's assistance, at each clinic visit. These toxicities are recorded and monitored by routine review of systems, clinical laboratory results, and other clinical assessment (i.e. chest x-rays, MUGAs, etc.). To date our toxicity reporting does not indicate any of our 36 subjects have developed an immune response to HER2. Specifically, the toxicities observed while may have included some of the above, the adverse events have not been unexpected (e.g. skin reactions are mostly injection site reactions) and/or sustained for prolonged periods of time during or post-vaccination.

***Task 3**: To determine the immunogenicity of a HER2 ICD peptide-based vaccine in patients with Stage IV breast cancer receiving concurrent trastuzumab monotherapy*

a. Determine the immunogenicity of the approach by assessing the T cell response to HER2 ICD. We have evaluated the T cell responses in sixteen patients. The PBMC obtained before and after vaccination were stimulated with the three ICD peptides included in this vaccine and overlapping peptide pools for the HER2 intracellular domain (ICDpm). The T cell responses were assessed using a standard 10 day IFN-gamma(g) ELISPOT assay. In this assay, the cells were stimulated with p776, p927 p1166 and ICDpm on Day 1, and re-stimulated on Day 8. The spots of IFN-g secreted after the stimulations were developed and counted on day 10 using an automated ELISPOT plate reader. Our results show that after the vaccination, the p776 specific response (HER2 specific cells/10^6 PBMN) increased 4.1 fold (pre vs. post: 99 ± 32 vs. 408 ± 96; mean ± SE; n=16. p=0.005), the p927 specific response increased 3.4 fold (pre vs. post: 162 ± 55 vs. 542 ± 134; p=0.013), and the p1166 response increased 3.5 fold (pre vs. post 182 ± 51 vs. 640 ± 130; p=0.003) after the vaccination (Fig. 1A). These patients also developed enhanced responses to ICDpm (pre vs. post: 473 ± 189 vs. 705 ± 136; n=16; p=0.328). In contrast, the response to tetanus toxoid (TT) did not obviously increase post vaccination (pre vs. post: 891 ± 147 vs. 1293 ± 200; p=0.116) (Fig. 1B). Among the sixteen patients, fourteen (88%) developed immunity to p776, twelve (75%) developed immunity to p927, thirteen (81%) developed immunity to p1166, and ten (69%) developed immunity to ICDpm (Fig. 2). The evaluation of T cell response to HER2 ICD on remaining subjects is on-going.

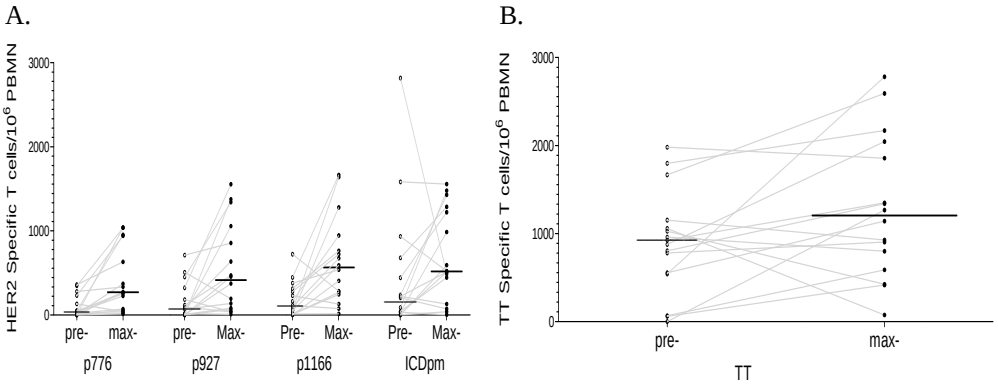

Figure 1. HER2 ICD peptide immunity elicited after the HER2 vaccination. A. HER2 antigen stimulated responses; B. TT antigen stimulated response. The bars indicate the median.

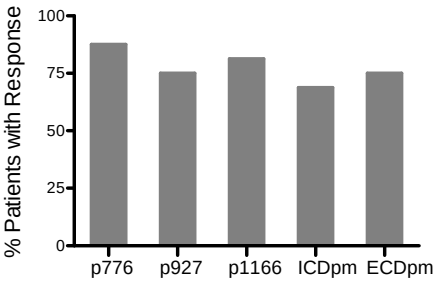

Figure 2. HER2 ICD peptide vaccine stimulated HER2 specific immunity in the majority of the patients.

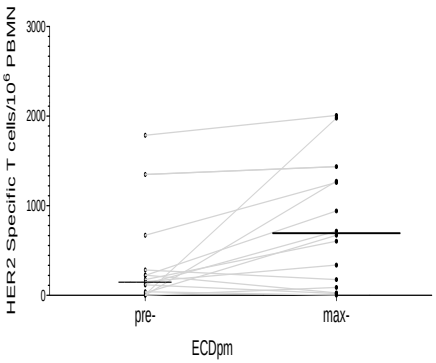

Figure 3. HER2 ECD immunity elicited after ICD peptide vaccination. The bars indicate the median.

b. Determine the incidence of epitope spreading to the HER2 ICD or other peptides in the immunizing mix (intermolecular epitope spreading). We have evaluated the T cell responses to overlapping peptide pools for the HER2 extracellular domain (ECD pm), which is not included in the vaccine. The immune responses generated against HER2 ECDpm represent epitope spreading. We found that patients developed IFNg-secreting Th1 responses to ECDpm (pre vs. post: 409 ± 142 vs. 811 ± 172; p=0.082) (Fig. 3). Among the sixteen patients, twelve (75%) developed epitope spreading (Fig. 2). Our group has demonstrated that the patient's survival was significantly associated with the development of epitope spreading following vaccination (Salazar L 2009). These data suggest that these patients may have improved survival benefit after the vaccination.

The assessment of the incidence of epitope spreading on remaining subjects is on-going.

Assess the serum level of TGFb. We continually evaluated the serum levels of TGF-beta (b) in patients before and after the vaccination using a human TGFb1 ELISA kit (eBioscience, San Diego, CA). TGF-b is an immunosuppressive cytokine secreted by tumor and immunosuppressive cells. We found that the levels of serum TGFb decreased in 11 of the 18 patients evaluated after the 3rd vaccination. The mean level of hTGFb was 1460 (± 37) pg/ml before the vaccination. It decreased to 984 (± 30) pg/ml after the 3rd vaccine, and maintained at 985 (± 25) pg/ml after 6th vaccine (mean ± SE, n=18). Thus, the mean level of serum TGFb decreased more than 33% after vaccination, although it did not reach statistical significance. The decreased levels of serum TGFb may predict a better prognosis as elevated levels of serum TGFb are associated with an increased risk of relapse in breast cancer patients (Bates GJ et al J Clin Oncol 2006). We will assess the serum levels of TGFb on remaining subjects when all specimens have been collected in order to decrease the variability among assays.

Correlate the serum TGFb with HER2 specific IFNg-secreting Th1 response. We analyzed the correlation between the change of serum levels of TGFb post vaccination and HER2 ICD vaccine-induced T cell response at the same time. We found that the greater the magnitude of HER2 specific T cell response, as demonstrated by IFNg secretion, the greater the decrease in serum TGFb. The increased T cell response to ICDpm correlated with decreased levels of TGFb (p=0.097, r=0.445, Fig. 4A). The correlation between increased epitope spreading T cell response and decreased levels of TGFb was significant (p=0.0397, r=0.535, Fig. 4B). In contrast, there was no correlation between the magnitude of TT response and the change in TGF-b levels (p=0.889; r=0.041, Fig. 4C). Thus, the increased numbers of HER2 antigen, especially HER2 ECD, elicited via HER ICD peptide vaccination was associated with a decrease in serum TGF-b levels. We will confirm this observation after we finish the above analysis on all subjects.

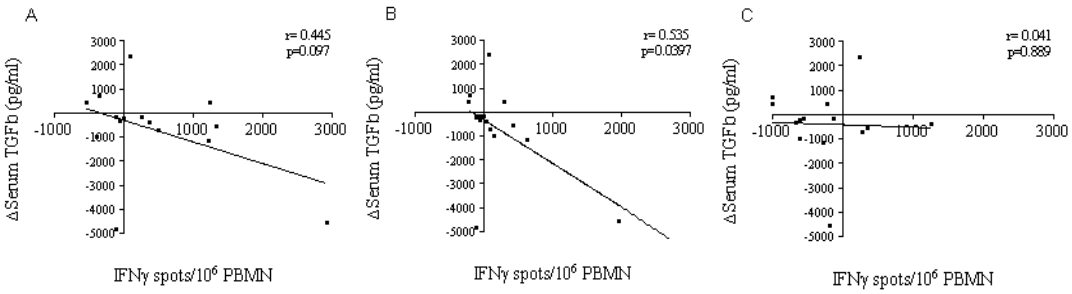

Figure 4. HER2 ICD peptide vaccine induced ICD peptide response (A) and epitope spreading (B), not tetanus toxoid response (C), after immunization were associated with a decrease in serum TGF-beta levels. X axis: IFNg secretion (post-pre vaccine); Y axis: serum levels of TGFb (Post-pre vaccine).

Evaluate multiple CD4 T cell cytokine responses induced by the vaccine. CD4+ T cell mediated immune responses to tumor antigens elicited after vaccinations are central to the efficacy of tumor vaccine. Multiple tumor specific Th1 (IFNg/TNFa/IL2), or Th1/Th17 cells may be superior to IFNg-secreting Th1 cells alone in the prevention of tumor relapse. Th17 is a subset of CD4 Th cytokine recently reported and has been found to have anti-tumor effects in tumor-bearing animals. We assessed the levels of multiple cytokines secreted from PBMN collected 1 month after the 3rd vaccine with those from pre-vaccinated PBMN as control. The supernatants collected on Day 8 after the antigen stimulation from the 10 day IFNg ELISPOT assays

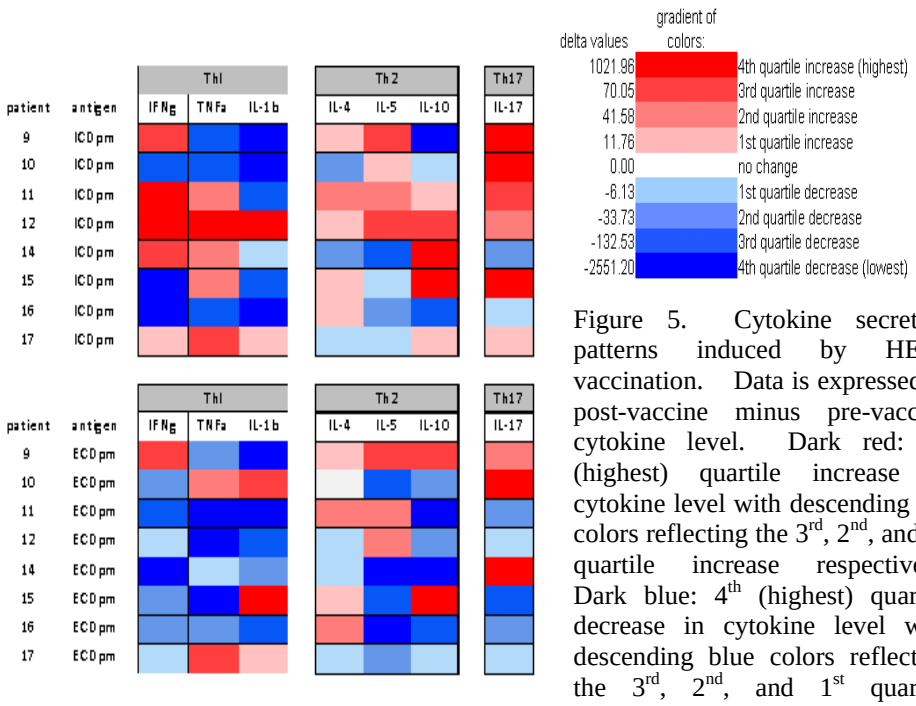

Figure 5. Cytokine secretion patterns induced by HER2 vaccination. Data is expressed as post-vaccine minus pre-vaccine cytokine level. Dark red: 4th (highest) quartile increase in cytokine level with descending red colors reflecting the 3rd, 2nd, and 1st quartile increase respectively. Dark blue: 4th (highest) quartile decrease in cytokine level with descending blue colors reflecting the 3rd, 2nd, and 1st quartile decrease respectively.

as described above were used for this assay. Simultaneous detection of multiple cytokines (IFN-γ, TNF-α, IL-1b, IL-4, IL-5, IL-10, and IL-17) was performed using a custom multiplex cytokine kit (Millipore) on Luminex. Figure 5 shows preliminary data from eight patients. Data collected via cytokine multiplexing is color coded as to the magnitude of antigen specific cytokine increase (red) or decrease (blue) with vaccination. Both ICDpm and ECDpm induced responses are shown. The data suggest several specific patterns of Th response to the HER2 antigens. Half of the patients increased IFNg/TNFa responses (Patients 11, 12, 14 and 17); half of the patients increased IFNg/IL17 and IFNg/Th17/Th2 responses (patient 9, 11, 12 and 17). In contrast, Patient 16's T cells decreased both Th1 and Th17 cytokine production after stimulation. We will further determine the levels of multiple cytokine secretion after the vaccination of all subjects and evaluate their significance in the prevention of tumor relapse.

c. Determine the incidence of epitope spreading to other immunogenic proteins associated with breast cancers (extramolecular epitope spreading). We will perform this analysis using available PBMN after we finish Tasks 3a and 3b.

d. Assess the absolute magnitude of the CD4+ and CD8+ HER2 specific immune responses generated after active immunization. We will also perform this analysis using available PBMN after we finish Tasks 3a and 3b.

e. Evaluate the generation of HER2 specific antibody immunity and antibody avidity. We have not been able to develop a HER2 antibody assays that would allow reproducible identification of HER2 specific antibody responses while patients are receiving trastuzumab (as trastuzumab interferes with the assay). These experiments will be considered on hold.

f. Determine whether overall survival is associated with the development of HER2 specific T cell response or epitope spreading after active immunization. Overall survival for the first 25 subjects will be complete November 2011 which is 2 years out from the last vaccine of the 25th subject. Relapse free survival, which is evaluated 4 years after the last vaccine for the last enrolled subject. Since we have not concluded enrollment we do not have a definite date of completion.

KEY RESEARCH ACCOMPLISHMENTS

- Completion of accrual to the study
- Interim analysis on first 25 subjects indicated a progression free survival benefit
- Demonstrated significant augmentation of immunity in vaccinated patients
- Demonstrated epitope spreading in the majority of patients evaluated to date

REPORTABLE OUTCOMES

- To date the toxicity profile consists of grades 1 and 2 demonstrating that the vaccine is safe to give with trastuzumab.
- To date there is no significant cardiac toxicity when combining the HER2 ICD vaccine with trastuzumab.

CONCLUSIONS

We are ending accrual to the study at 38 subjects rather than 52 subjects. This will still give us enough statistical power to answer our primary objective to evaluate relapse free survival.

The toxicity profile for this study suggests that this vaccine given in combination with trastuzumab is safe with only grades 1 and 2 toxicities.

PUBLICATIONS

1. Mary L. Disis, Danelle R. Wallace, Theodore A. Gooley, Yushe Dang, Meredith Slota, Hailing Lu, Andrew L. Coveler, Jennifer S. Childs, Doreen M. Higgins, Patricia A. Fintak, Corazon dela Rosa, Kathleen Tietje, John Link, James Waisman, and Lupe G. Salazar. Concurrent Trastuzumab and HER2/neu-Specific Vaccination in Patients With Metastatic Breast Cancer. J Clinical Oncology 27(28):4685-92.

2. Mary Disis, Lupe G. Salazar, Doreen Higgins, Jennifer Childs, Miriam Bolding, Becky Royer, Danelle Wallace, Yushe Dang, Patricia A. Fintak, and James R. Waisman. Phase II Study of a HER-2/neu Peptide-Based Vaccine Plus Concurrent Trastuzumab for Prevention of Breast Cancer Relapse (BC030289). Era of Hope, 2009.

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ORIGINAL REPORT

Concurrent Trastuzumab and HER2/*neu*-Specific Vaccination in Patients With Metastatic Breast Cancer

Mary L. Disis, Danelle R. Wallace, Theodore A. Gooley, Yushe Dang, Meredith Slota, Hailing Lu, Andrew L. Coveler, Jennifer S. Childs, Doreen M. Higgins, Patricia A. Fintak, Corazon dela Rosa, Kathleen Tietje, John Link, James Waisman, and Lupe G. Salazar

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Clinical Trials repository link available on JCO.org.

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The Acknowledgment is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

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ABSTRACT

Purpose
The primary objectives of this phase I/II study were to evaluate the safety and immunogenicity of combination therapy consisting of concurrent trastuzumab and human epidermal growth factor receptor 2 (HER2)/*neu*-specific vaccination in patients with HER2/*neu*-overexpressing metastatic breast cancer.

Patients and Methods
Twenty-two patients with stage IV HER2/*neu*-positive breast cancer receiving trastuzumab therapy were vaccinated with an HER2/*neu* T-helper peptide-based vaccine. Toxicity was graded according to National Cancer Institute criteria, and antigen specific T-cell immunity was assessed by interferon gamma enzyme-linked immunosorbent spot assay. Data on progression-free and overall survival were collected.

Results
Concurrent trastuzumab and HER2/*neu* vaccinations were well tolerated, with 15% of patients experiencing an asymptomatic decline in left ventricular ejection fraction below the normal range during combination therapy. Although many patients had pre-existing immunity specific for HER2/*neu* and other breast cancer antigens while treated with trastuzumab alone, that immunity could be significantly boosted and maintained with vaccination. Epitope spreading within HER2/*neu* and to additional tumor-related proteins was stimulated by immunization, and the magnitude of the T-cell response generated was significantly inversely correlated with serum transforming growth factor beta levels. At a median follow-up of 36 months from the first vaccine, the median overall survival in the study population has not been reached.

Conclusion
Combination therapy with trastuzumab and a HER2/*neu* vaccine is associated with minimal toxicity and results in prolonged, robust, antigen-specific immune responses in treated patients.

J Clin Oncol 27:4685-4692. © 2009 by American Society of Clinical Oncology

INTRODUCTION

A potential application of cancer vaccines is in the prevention of tumor recurrence or progression in patients with minimal residual disease. Vaccines will need to be coadministered with primary therapy or given after optimal treatment. Human epidermal growth factor receptor 2 (HER2)/*neu* is a tumor antigen and vaccine target in breast cancer. With the prolonged use of trastuzumab in the treatment of most HER2/*neu*-positive breast cancers, evaluation of the potential additive toxicity of the combination of trastuzumab and HER2/*neu* vaccination is warranted.

The vaccine used in this trial was designed to elicit HER2/*neu*-specific T-helper (Th) immunity.[1] Vaccine-induced $CD4^+$ Th1 cells may traffic to the tumor, secrete inflammatory cytokines such as interferon gamma (IFN-γ), and activate local antigen-presenting cells (APC) enhancing cross-priming at the tumor site.[2] Via cross-priming, tumor-specific $CD8^+$ T cells can be elicited.[3] Finally, antigen-specific $CD4^+$ T cells can enhance and sustain tumor-specific T-cell immunity over time.

Data presented here suggest that combination trastuzumab and HER2/*neu* vaccine therapy is safe and generates robust and persistent tumor-specific T-cell immunity.

PATIENTS AND METHODS

Patient Population

After informed consent, patients were enrolled in this trial approved by the United States Food and Drug

Administration and the University of Washington Human Subjects Division. Enrollment criteria were as follows: stage IV breast cancer in complete remission (CR) or stable disease (SD) on trastuzumab; documented HER2/*neu* overexpression via immunohistochemistry or fluorescent in situ hybridization; HLA-$A2^+$; and a left ventricular ejection fraction (LVEF) in the normal range (Table 1). Twenty-two patients were enrolled, and 21 patients received vaccinations. Fourteen of 21 patients completed all six immunizations; five of 21 patients completed at least three immunizations, a sufficient number to immunize; and two of 21 patients received fewer than three immunizations.[4] Clinical data are presented on 21 patients. Immunologic data for at least one immunizing peptide and protein are presented on patients who had baseline and at least one additional evaluation of immunity assessed (n = 19).

Table 1. Patient Characteristics

Characteristic	No. of Patients	%
Intent to treat	22	
Received treatment	21	
Age, years		
Median	49	
Range	33-76	
Time from metastatic diagnosis, months		
Median	18	
Range	7-76	
Disease status		
Complete remission	11	52
Stable	10	48
Time from last chemotherapy, months		
Median	4	
Range	1-61	
No. of prior chemotherapy regimens		
< 2	6	29
2-3	12	57
≥ 4	3	14
Time on trastuzumab before study entry, months		
Median	13	
Range	3-85	
LVEF at time of study entry		
Median	61	
Range	46-72	
ER status		
Positive	13	62
Negative	8	38
PR status		
Positive	9	43
Negative	12	57
Prior hormonal therapy		
Yes	15	71
No	6	29
Concurrent hormonal therapy with vaccination		
Yes	7	33
No	14	67
HER2 status by IHC		
2+	5	24
3+	15	71
Unknown	1	5
HER2 status by FISH, n = 12		
Median	5.18	
Range	2.05-12.34	

Abbreviations: LVEF, left ventricular ejection fraction; ER, estrogen receptor; PR, progesterone receptor; IHC, immunohistochemistry; FISH, fluorescent in situ hybridization.

Study Design

Serum and peripheral-blood mononuclear cells (PBMCs) were collected before, at midpoint, and at 1, 3, 6, and 12 months after immunization. The sample size was chosen based on the following: if no toxicities were seen, the probability of such an occurrence would be at least 90% if the true toxicity rate was 10% or less.

T-Cell Responses

IFN-γ enzyme-linked immunosorbent spot assay was performed as previously described.[5] Ten μg/mL of immunizing peptides were used: p369 through 384 (KIFGSLAFLPESFDGDPA) derived from the extracellular domain (ECD), p688 through 703 (RRLLQETELVEPLTPS) from the transmembrane domain, and the intracellular domain (ICD) derived p971 through 984 (ELVSEFSRMARDPQ; denoted as 15 [eg, "X."15] or 9 [eg, "X."9] amino acids in length).[1,6] Each antigen was assessed in six replicates of 2×10^5/well. One μg/mL of overlapping peptide pools (15 amino acids overlapping by 11 amino acids) for the HER2/*neu* ICD or ECD, 1 μg/mL of recombinant human p53, insulin-like growth factor binding protein 2 (IGFBP-2; Sigma-Aldrich, St Louis, MO), and topoisomerase II-α (Topogen, Columbus, OH) were also used. Tetanus toxoid (0.5 U/mL) and phytohemaglutinin (2.5 ug/mL) were positive controls. All samples for each patient were cryopreserved, then thawed and analyzed simultaneously to ensure comparability.[7,8] Validation studies demonstrated that the assay is linear and precise between 2.0 and 3.5×10^5 PBMCs/well, with a detection limit of 1:100,000. Data are calculated estimates, and some results are considered below the level of reproducible detection. Ten age-matched volunteer female donors were evaluated as controls. Data are presented on individuals as a calculated 1/frequency of IFN- γ–secreting cells in 10^6 PBMCs and discussed as the ratio of responding cells to PBMCs. In summary analyses, data are presented as IFN-γ spots per well (SPW) corrected for background or described as the number of spots per 10^6 PBMCs postvaccination minus the number of spots prevaccination.

Patients were considered to have pre-existing immunity if, at baseline, the mean antigen-specific SPWs were statistically different ($P < .05$) from no antigen wells. Patients were considered to have increased response if the current SPW was greater than 2 standard deviations (SD) above the previous value, remained the same if the mean SPW was within 2 SD of the previous value, and decreased if the mean SPW was greater than 2 SD below the previous value. Two SD is equivalent to a P value of .05 in that there is a 95% probability that the values are statistically significant.[9]

Cytolytic function of generated T-cell lines[5] was evaluated (Cytotox 96, Promega, Madison, WI). The HLA-A2 transfected human HER2/*neu*-expressing breast cancer cell line, SKBR3-A2, was plated at 10^4 cells/well in triplicate. T cells, expanded after stimulation with immunizing peptide, were added in an effector/target ratio of 40:1. Nontransfected SKBR3 cells were controls. After incubation, supernatants were analyzed per manufacturer's specifications. Percent specific lysis was calculated as: ([experimental release − spontaneous releases of cytotoxic T-lymphocyte cells and target cells]/[maximum release − spontaneous release of target cells]) × 100.

Serum Transforming Growth Factor Beta Levels

Levels were measured, in triplicates, by enzyme-linked immunosorbent assay (eBioscience, San Diego, CA). The concentration of human transforming growth factor beta (TGF-ß) was calculated from a curve of serially diluted human recombinant TGF-ß. The change in TGF-ß levels is described as the value of TGF-ß postvaccination minus the value of TGF-ß prevaccination in picograms per milliliter.

T-Regulatory Cell Levels

Evaluation was performed as previously described.[10] Data are expressed as the percentage of $FOXP3^+CD4^+$ cells among all $CD4^+CD3^+$ T cells.

Statistical Analysis

Differences in median immune responses were assessed using a two-tailed Mann-Whitney test, with a level significance set at .05. The relationship between magnitude of immunity and serum TGF-ß levels was assessed using Pearson's product moment correlation. Pre- versus postimmunization data were compared using a paired t test (two-tailed). Kaplan-Meier curves were

generated to show the probability of overall survival (OS) and progression-free survival (PFS), where OS was defined as the time elapsed between beginning vaccinations and death or last follow-up, and PFS was defined as the time elapsed between first vaccine and the earliest of death, disease progression as reported by the patients' primary physicians, or last contact. Data for patients without death (for OS) or death as a result of progression (for PFS) were censored at the date of last known status. Differences in survival curves based on immunologic response were assessed by the Gehan-Breslow-Wilcoxon test. Analyses were performed with GraphPad InStat v.5.01 (GraphPad Software, San Diego, CA).

RESULTS

*HER2/*neu *Th Peptide Vaccine Administered Concurrently With Trastuzumab Was Well Tolerated and Did Not Result in Additional Cardiac Toxicity*

Table 2 details 573 adverse events. The majority of toxicities were grade 1 or 2 (99%). There were four grade 3 toxicities, three possibly related to the treatment: injection-site reaction, fainting, and ulceration. There was one nonrelated grade 4 event, a stroke. The median LVEF before treatment was 61% (range, 46% to 72%) and post-treatment was 61% (range, 45% to 66%). Three patients (15%) had a decrease in LVEF to less than normal values on study. None developed symptoms of left ventricular dysfunction. Cardiac toxicities were grade 1 and 2.

*HER2/*neu *Th Peptide Vaccine Administered Concurrently With Trastuzumab Stimulates or Boosts HER2/*neu*-Specific Immunity in the Majority of Patients*

The median peptide-specific T-cell response before the first vaccine was a frequency of less than 1 antigen-specific cell in 75,000 PBMCs (range, 1:400,000 to 1:77,000; Fig 1A). Ninety percent of patients developed new or augmented immunity. The maximal response to p369.15 was a median frequency of one in 4,121 PBMCs (range, 1:323 to 1:2,000,000; $P = .0015$ compared with prevaccination), the maximal response to p688.15 was one in 4,152 PBMCs (range, 1:307 to 1:3,000,000; $P = .0012$), and the maximal response to p971.15 was one in 2,086 PBMCs (range, 1:266 to 1:86,960; $P = .0066$; Fig 1A). Ten (53%) of 19 patients had pre-existing immunity to any of these peptides. Sixteen patients (84%) significantly augmented immunity, three (16%) did not augment, and none had a decrease in peptide-specific immunity with immunization. The percentage of responding patients for the peptides is shown (Fig 1B).

Table 2. Adverse Events

Adverse Event	AE		Possibly, Probably, or Definitely Related	
	No.	% of All AEs	No.	% of All Related AEs
Most common				
Injection site reaction	64	11	64	17
Fatigue	42	7	38	10
Myalgias	42	7	33	9
Headache	41	7	27	7
Lymphopenia	33	6	28	7
Leukocytes, total WBCs	29	5	27	7
Pruritus/itching	21	4	19	5
Nausea	20	3	13	3
Rigors/chills	20	3	16	4
Diarrhea	16	3	5	1
AE grading*				
1	508	89	337	88
2	60	10	43	11
3	4	1	3	1
4	1	0	0	0
5	0	0	0	0
Cardiac AEs				
Palpitations	9	45	2	29
Hypertension	5	25	0	0
Left ventricular systolic dysfunction	3	15	3	43
Chest tightness	1	5	0	0
Supraventricular and nodal arrhythmia	1	5	1	14
Other	1	5	1	14
AE grading*				
1	12	60	1	14
2	8	40	6	86
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0

Abbreviation: AE, adverse event.
*For AE grading, percent is shown (not % of all AEs or % of all related AEs).

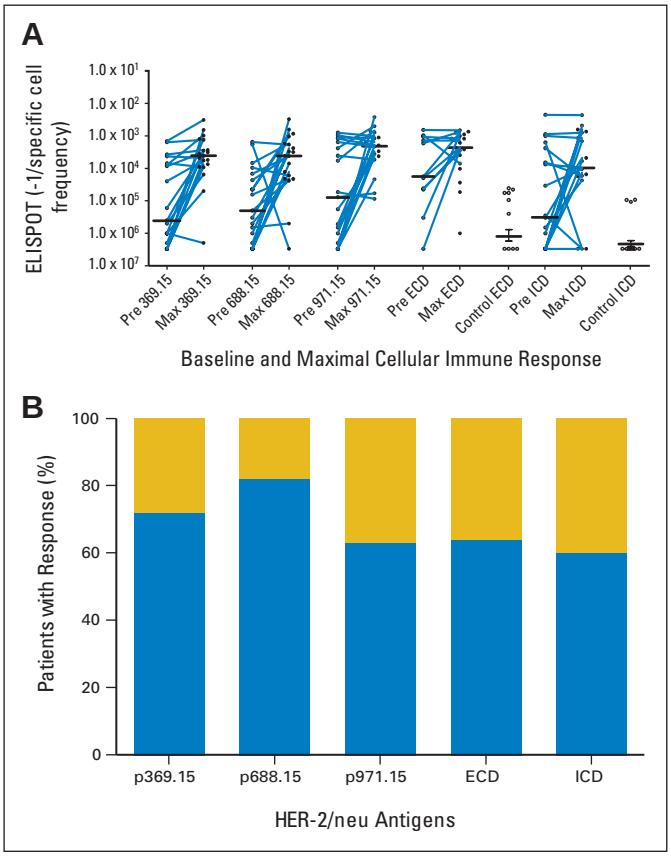

Fig 1. A human epidermal growth factor receptor 2 (HER2)/*neu* T-helper peptide vaccine administered concurrently with trastuzumab stimulates or boosts HER2/*neu*-specific immunity in the majority of patients. (A) Prevaccine (Pre) and maximal (Max) responses 1/frequency (*Y* axis) tested antigens (*X* axis). Connected points: mean and SE of six replicates with median bar. Data derived from 10 controls are shown for extracellular domain (ECD) and intracellular domain (ICD). (B) Percent HER2/*neu*-specific immunity after vaccination. Blue, percent increased; yellow, percent unchanged. ELISPOT, enzyme-linked immunosorbent spot assay.

Peptides were derived from both the HER2/*neu* ECD and ICD. Of note, seven (64%) of 11 patients had significant pre-existing immunity to the ECD (median, 1:17,729; range, 1:660 to 1:3,000,000), and six (32%) of 19 patients had significant pre-existing immunity to the ICD (median, 1:309,524; range, 1:226 to 1:3,000,000; Fig 1A). Sixty-nine percent of patients developed new or augmented immunity: 37% to ECD, 53% to ICD, and 21% to both. The maximal response to the ECD was a median of one cell in 2,312 PBMCs (range, 1:678 to 1:1,000,000; $P = .3017$ compared with baseline) and to the ICD was one cell in 9,677 PBMCs (range, 1:232 to 1:3,000,000; $P = .0894$; Fig 1A). Five patients did not augment, and none had a significant decrease in domain immunity with immunization. The percentage of responding patients is shown (Fig 1B).

Vaccination Can Elicit Tumor-Specific Cytotoxic T Cells

Embedded within the native sequence of the Th peptides are HLA-A2 binding motifs: p369.9, p688.9, and p972.9.[1,5] The maximal response to class I peptides was a median frequency to p369.9 of 1:10,200 (range, 1:1,032 to 1:3,000,000; $P = .0030$ compared with baseline), to p689.9 was 1:10,050 (range, 1:1,039 to 1:3,000,000; $P = .1720$), and to p971.9 was 1:5,659 (range, 1:845 to 1:3,000,000; $P = .0126$; Fig 2A). T-cell cultures were established for five patients who had excess PBMCs available. The resultant T-cell lines could specifically lyse HER2/*neu*-positive/HLA-A2–positive breast cancer cells (range, 7% to 70% lysis; Fig 2B).

Seven (37%) of 19 patients had pre-existing immunity to these HLA-A2 peptides. Overall, 14 patients (74%) significantly augmented the class I HER2/*neu* peptide-specific immune response, four patients (21%) did not augment, and one patient had a decrease in immunity

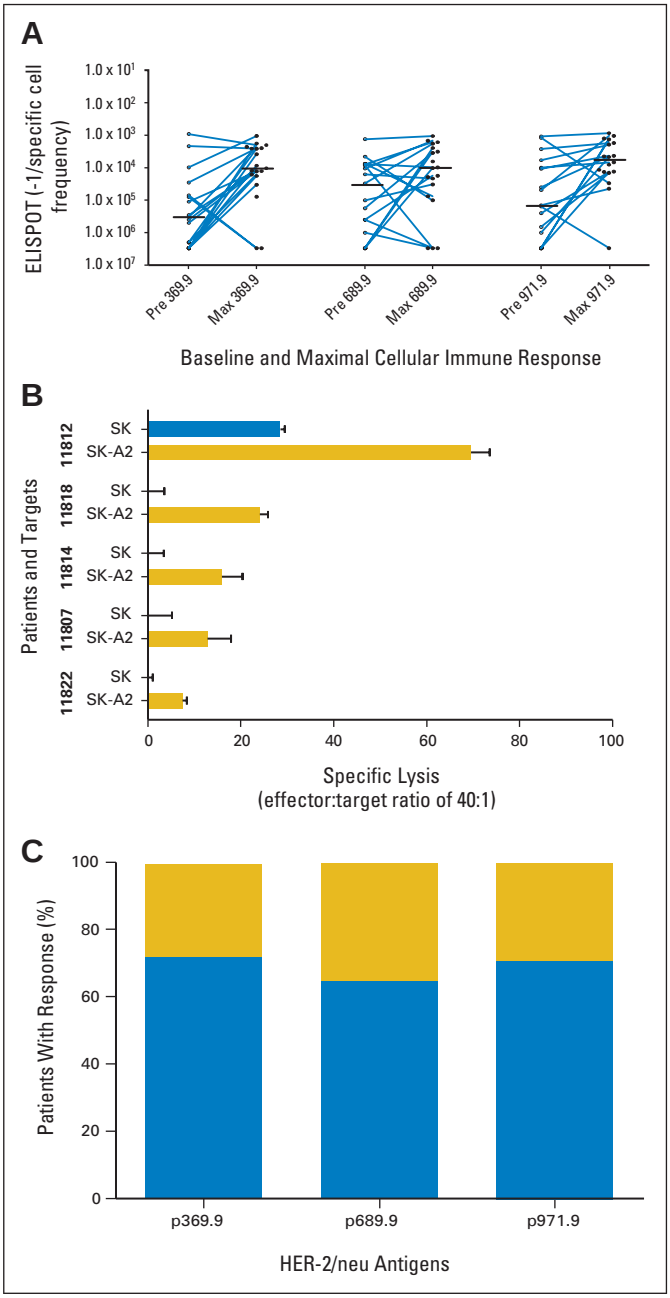

Fig 2. Vaccination can elicit tumor-specific cytotoxic T cells. (A) Prevaccine (Pre) and maximal (Max) responses 1/frequency (*Y* axis) HLA-A2 peptides (*X* axis). Connected points: mean and SE of six replicates with median bar. (B) Percent lysis: SKBR3 (blue), SKBR3-A2 (yellow) with SE of four replicates. (C) Percent human epidermal growth factor receptor 2 (HER2)/*neu* peptide-specific immunity. Blue, percent increased; Yellow, percent unchanged. ELISPOT, enzyme-linked immunosorbent spot assay.

to the peptides with immunization. Percentage of responding patients for the HLA-A2 peptides is shown (Fig 2C).

Vaccination-Induced or Enhanced Epitope Spreading Was Observed in the Majority of Patients and Was Associated With a Decrease in Serum TGF-ß

p98.15 and p776.15 are native epitopes of HER2/*neu*, immunogenic, and not included in the vaccine formulation.[11] Development of immunity to these epitopes demonstrates intramolecular epitope spreading, which was elicited or augmented in the majority of patients (Fig 3A).[6] The median maximal T-cell response to nonimmunizing epitopes was a frequency to p98.15 of one in 7,558 PBMCs (range, 1:1,205 to 1:3,000,000; $P = .0055$ compared with prevaccination) and to p776.15 of one in 2,183 PBMCs (range, 1:527 to 1:40,000; $P = .0006$; Fig 2A). Nine (47%) of 19 patients had pre-existing immunity to these peptides. Fourteen patients (74%) significantly augmented the immune response, five patients (26%) did not augment, and none significantly decreased immunity to these peptides with immunization.

We have identified several immunogenic breast cancer–associated proteins and questioned whether new or augmented immunity to IGFBP-2, p53, and topoisomerase II-α were stimulated with vaccination (ie, intermolecular epitope spreading).[10,12] Seven patients had sufficient PBMCs available for this analysis. All patients had a pre-existing immune response to at least one antigen, and all seven developed new or augmented immunity to at least one of the antigens (Fig 2B). The postvaccination median response to IGFBP-2 was a frequency of one in 5,405 PBMCs (range, 1:1,993 to 1:150,000; $P = .0973$ compared with prevaccination), postvaccination median response to p53 was one in 6,793 PBMCs (range, 1:1,061 to 1:109,091; $P = .1282$), and postvaccination median response to topoisomerase II-α was one in 3,659 PBMCs (range, 1:1,575-1:8,219; $P = .0111$; Fig 2B). Five patients (71%) augmented immunity to IGFBP-2, six patients (86%) augmented immunity to p53, and all patients tested augmented immunity to topoisomerase II-α. Two patients had a significant decrease in a pre-existing immune response to IGFBP-2 with immunization.

The multiple specificities of IFN-γ secreting T cells induced by vaccination led us to question whether these T cells, which could potentially traffic to tumor, might impact the immunosuppressive environment that has been described in breast cancer.[13,14] TGF-ß has been shown to be elevated in the serum of patients with breast cancer and is associated with T-cell dysfunction.[15-17] The greater the magnitude of the intramolecular epitope spreading T-cell response, the greater the decrease in serum TGF-ß ($r = 0.614$; $P = .0003$; Fig 3C). There was weak correlation between the magnitude of the tetanus toxoid response, evaluated as a control, and change in TGF-ß levels ($r = 0.016$; $P = .93$; Fig 3D).

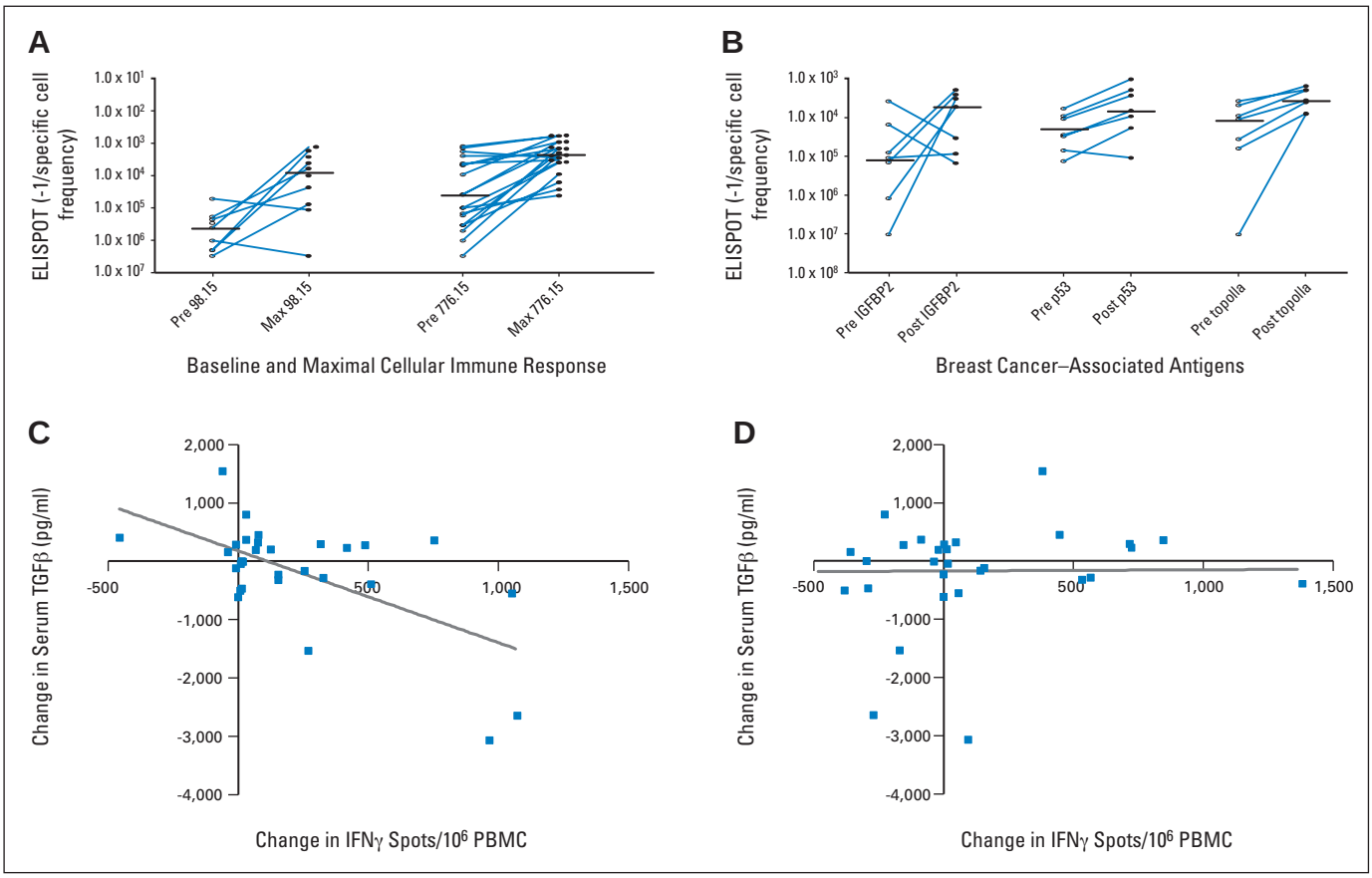

Fig 3. Vaccination-induced epitope spreading occurred in the majority of patients and was associated with a decrease in serum transforming growth factor beta (TGF-β) levels. (A) Prevaccine (Pre) and maximal (Max) intramolecular epitope spreading (IMS) 1/frequency (*Y* axis) peptides (*X* axis). (B) Pre-Max intermolecular epitope spreading (*Y* axis) antigens (*X* axis). Connected points: mean and standard deviation of six replicates with median bar. (C) *X* axis, change in magnitude of IMS response (interferon gamma [IFN-γ] spots per well/10^6 peripheral-blood mononuclear cells [PBMCs]). (D) *X* axis, change in magnitude of tetanus toxoid response. *Y*-axis; change in TGF-ß. ELISPOT, enzyme-linked immunosorbent spot assay.

Treg levels were measured before and after immunization in eight patients. The median percent Treg was 1.64% before immunization (range, 0.33% to 7.33%) and 1.32% 1 month after vaccines (range, 0.41% to 5.08%; $P = .60$). At 1 year after immunization, the median Treg level was 1.11% (range, 0.24% to 4.91%; $P = .61$ compared with preimmunization).

HER2/neu-Specific Immunity Can Persist and Even Increase After Active Immunizations Have Ended

Figure 4 shows the OS and PFS of the study population from the time of first vaccination. The median follow-up among survivors was 36 months (range, 21 to 49 months). The median PFS was 17.7 months, and the Kaplan-Meier estimate of PFS was 33% at 3 years. The median OS has not been reached; the Kaplan-Meier estimate of OS is 86% at 4 years.

Although the generation of a new or augmentation of a pre-existing immune response to either immunizing peptides or protein ($P = .11$) or to epitope spreading peptides ($P = .47$) was not associated with survival, the magnitude of immunity generated tended to be higher in surviving patients. All 10 patients who had T-cell responses greater than the median to HER2/*neu* immunizing peptides and associated protein ($P = .08$) or to peptides associated with intramolecular epitope spreading ($P = .09$) were survivors as compared with patients with responses less than the median.

We monitored immunity to HER2/*neu*-related antigens after the end of immunizations in 11 patients (ECD, n = 6). Five patients (46%) maintained the same level of immunity to p369.15 in long-term follow-up as compared with the maximal response achieved during active immunization. Two (18%) decreased immunity and four (36%) continued to augment immunity (median, 1:4,121; range, 1:788 to 1:3,000,000). Seven patients (64%) maintained the same level of immunity to p688.15. One patient (9%) decreased compared with the maximal response achieved, and three patients (27%) continued to augment immunity to p688.15 (median, 1:12,876; range, 1:307 to 1:3,000,000). Five patients (46%) maintained the same level of immunity to p971.15. Two patients (18%) decreased and four patients (36%) continued to augment immunity (median, 1:5,236; range, 1:266 to 1:222,222).

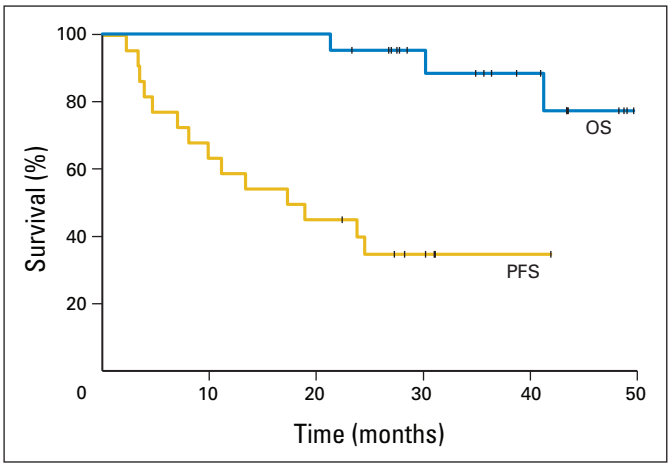

Fig 4. Overall survival (OS) and progression-free survival (PFS) of immunized patients. Kaplan-Meier curves of the percent OS and PFS in months from the time of first vaccine (n = 21).

Persistent immunity to the HER2/*neu* ECD and ICD was assessed. Five (83%) of six patients maintained the same level of immunity to the ECD in follow-up. One patient (17%) had decreased and none continued to augment immunity to the ECD (median, 1:4,625; range, 1:803 to 1:11,321). Six (55%) of 11 patients maintained immunity to the ICD. Two (18%) decreased and three (27%) continued to augment immunity (median, 1:6,024; range, 1:232 to 1:3,000,000). Finally, intramolecular epitope spreading was maintained in five patients (46%) as evidenced by immunity to p776.15. Two patients (18%) significantly decreased and four (36%) continued to augment immunity (median, 1:2,667; range, 1:527 to 1:300,000).

DISCUSSION

Concurrent administration of trastuzumab and a HER2/*neu*-specific vaccine is tolerated in patients with metastatic breast cancer (MBC), and significant pre-existing immunity to HER2/*neu* can be boosted and maintained with immunization. Moreover, epitope spreading elicited with vaccination may modulate systemic mediators of tumor-induced immune suppression.

Trastuzumab-related cardiac damage is generally reversible once the drug is stopped.[18] After immunization, however, the T-cell response is not as easily "turned off." As long as antigen is present, T-cells will clonally expand, further augmenting immunity, as occurred in a third of our patients. A study of long-term trastuzumab use in MBC reported that approximately 25% of patients experience some cardiac event.[18] In our trial, 15% of patients had an asymptomatic drop in left ventricular function below the normal range, although given the limited sample size, we cannot rule out the possibility that the true rate is higher than the historical rate.

An unexpected finding was the number of patients treated with trastuzumab who had significant pre-existing immunity to HER2/*neu* and other antigens. Although we do not have an assessment of HER2/*neu* immunity before starting trastuzumab, investigations by our group suggest only 10% of trastuzumab-naïve patients would have measurable cellular immunity.[19] Only one study has shown that endogenous HER2/*neu*-specific humoral and T-cell immunity could be elicited with trastuzumab treatment.[20] Despite the presence of immunity to HER-2/*neu* at the start of immunization, most responses could be boosted to greater levels with vaccination, and in patients with no pre-existing immunity, robust T-cell responses could be generated.

Priming with trastuzumab and boosting with an HER2/*neu* vaccine may generate levels of immunity more robust than vaccination alone. Indeed, the magnitude of the response achieved after immunization in this trial seemed to be greater than what we have historically observed. The patients in this study were comparable to the patients in our initial study both demographically and clinically.[1] There were no statistically significant differences in the two populations in age, disease status at time of vaccination, hormone receptor status, or number of chemotherapy regimens before vaccination. Our initial study did not include trastuzumab, as the drug was not in widespread use at the time, and the median peptide-specific T-cell response after vaccination was one in 16,129 PBMCs.[1] In this current study, the median T-cell response to all HER2/*neu* antigens was 1:1,838 PBMCs, a log-fold increase. Moreover, in our initial study, none of the patients continued to augment immunity after vaccinations had ended.[1] Here, nearly one third of patients augmented immunity, only a minority

demonstrated a diminution in response, and the remainder maintained the same level of HER2/*neu* immunity long term as during active immunization.

Vaccination induced epitope spreading. Epitope spreading is associated with autoimmune disease and enhances tissue destruction.[21,22] Some studies suggest that antibody-dependent cell-mediated cytotoxicity, by which APC presentation of new antigens is increased, is responsible for epitope spreading.[23,24] Others suggest that antigen-homing Th1-cells deliver cytokines, such as interferon, to the tumor, which activate local APC-enhancing cross-priming.[2,22] The presence of immunity to HER2/*neu* and other antigens before vaccination suggests that antibody-dependent cell-mediated cytotoxicity mediated by trastuzumab may have initiated cross-priming at the tumor site. The observation that increasing numbers of Th1 antigen-specific T cells, associated with epitope spreading and elicited via vaccination, decrease serum TGF-ß indicate that trafficking T cells may also be effectively modulating the tumor microenvironment. Theoretically, a decrease in serum TGF-ß could facilitate the continued augmentation and persistence of tumor-specific immunity.

Is HER2/*neu*-specific immunity induced by vaccination related to clinical outcome? Although the study was not designed to address a clinical end point, OS was assessed to gather additional data on the potential therapeutic efficacy of the combination.[25] The current observed results are encouraging in light of the historical results for patients with pretreated HER2/*neu*-positive MBC. Recent phase II studies using trastuzumab and various chemotherapy agents in the salvage setting have demonstrated that 40% to 60% of patients are able to achieve either complete or partial responses with second- or third-line trastuzumab-containing regimens. The median PFS and OS ranged from 7 to 12 and 18 to 23 months, respectively.[26-28] Combination therapy with trastuzumab and an HER2/*neu*-specific vaccine warrants further evaluation as a therapeutic regimen.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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PHASE II STUDY OF A HER-2 NEU PEPTIDE-BASED VACCINE PLUS CONCURRENT TRASTUZUMAB FOR PREVENTION OF BREAST CANCER RELAPSE

BC030289

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Background: Breast cancer relapse after optimal therapy is common in patients with HER-2/neu (HER2)-positive tumors and is likely due to residual microscopic disease. One approach to the eradication of residual subclinical disease is tumor vaccines that generate tumor-specific T cell immunity, specifically, memory T cells capable of eradicating tumor antigen-bearing cells over an extended period of time. Immunity against the intracellular domain (ICD) of the HER2 protein correlates with antitumor responses in animal models. Patients with HER2+ cancers can be immunized to the HER2 ICD using peptide-based vaccines. Moreover, trastuzumab, a standard therapy for HER2+ patients, increases the activity of HER2-specific T cells in vitro. Thus, concurrent administration of trastuzumab with HER2 vaccines may enhance the generation of HER2-specific CD4+ and CD8+ T cell responses and potentially translate into improved overall survival (OS) for advanced-stage patients. We have initiated a Phase II study to examine the OS, safety, and immunogenicity of an HER2 ICD peptide-based vaccine when administered concurrently with trastuzumab to patients with Stage IIIB/IV breast cancer.

Methods: A total of 52 subjects with HER2+ Stage IIIB or IV breast cancer who are currently on maintenance trastuzumab and have been treated to a state of no evidence of disease (NED) or stable bone-only disease (SBD) with trastuzumab alone or in combination with chemotherapy will be enrolled. Subjects are enrolled within 6 months of initiating maintenance trastuzumab and must have a normal baseline MUGA scan. The HER2 ICD peptide vaccine is composed of 3 HER2 Class II epitopes (p776-790, p927-941, and p1166-1180), and given intradermally with GM-CSF as adjuvant every 30 days for a total of 6 vaccines. The primary end point is evaluation of OS at 2 years compared to historical controls. Secondary end points include immunogenicity and safety. HER2-specific immune responses are assessed by IFN-γ ELISPOT at baseline and after vaccine 3 and 6. Toxicity is assessed at baseline prior to each vaccine and at follow-up.

Results: A total of 9 subjects have been enrolled to date, 7 Stage IV (5 NED and 2 SBD) and 2 stage IIIB. Median time from last chemotherapy was 4 months (range 1-11). A total of 7 subjects have completed 6 vaccines, and a total of 51 vaccinations have been given. Toxicities observed are grade I and II (82% and 17%, respectively) with the most common being fatigue (9%), myalgia (8%), leukopenia (8%), and lymphopenia (8%). There have been no related Grade 3 or 4 toxicities and specifically, no cardiac toxicities. At interim analysis, 3 of 4 patients have developed T cell immunity, defined as HER2-specific T cell precursors:PBMC) to p776, p927, and p1166. The median precursor frequency to p776 was 1:3,937 (range 1:1,051-1:20,000), to p927 was 1:1,574 (range 1:727-1:32,467), and to p1166 was 1:1483 (range 1:610-1:37,453). Interim survival data and complete immunologic analysis will be presented on patients enrolled to date.

Conclusions: Early data suggest that subjects with HER2+ stage IIIB and stage IV cancer can be safely immunized with an HER2 peptide vaccine while receiving concurrent trastuzumab. Additionally, the approach is immunogenic, generating significant levels of HER2-specific T cell immunity. Accrual continues and long-term follow-up is ongoing for survival benefit analysis.

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Treatment - Her2-Targeted Therapy

Phase II Study of a HER-2/Neu (HER2) Intracellular Domain (ICD) Vaccine Given Concurrently with Trastuzumab in Patients with Newly Diagnosed Advanced Stage Breast Cancer.

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HER2 is a tumor antigen in breast cancer and several trials have demonstrated that breast cancer patients can be immunized against this protein. We have developed HER2 peptide based vaccines that are aimed at eliciting $CD4^+$ Th1 tumor antigen specific T cell responses. Th1 effectors provide immunologic memory, enhance cross priming which will allow the elaboration of tumor specific $CD8^+$ T cells, and stimulate epitope spreading which we have shown to be a potential biomarker of clinical response. 52 patients will be enrolled with the primary objective to determine relapse free survival after active immunization. Eligible patients are newly diagnosed with Stage III (B or C) or Stage IV breast cancer and begin vaccination within 6 months of starting maintenance trastuzumab. This interim report will present data on the first 25 patients enrolled; 21 stage IV and 4 locally advanced patients. The vaccine is well tolerated with all adverse events (AE) being Grade I or 2. The most common AE is injection site reaction. Moreover, the combination of HER2 vaccination with trastuzumab did not result in additive cardiac toxicity in these patients. Immune responses were evaluated by IFN-gamma ELISPOT. To date, 88% of patients immunized developed significant immunity to the components of the ICD vaccine. The majority, 75%, developed robust immunity to the HER2 protein. Our group has recently demonstrated that a broadening of immunity throughout the HER2 protein, to components of the protein that weren't in the vaccine, i.e. epitope spreading, may be associated with improved survival in vaccinated patients. 63% of immunized patients demonstrated evidence of intramolecular epitope spreading. We questioned whether such high frequencies of homing Type 1 T cells might modulate the immunosuppressive tumor microenvironment, so we evaluated whether circulating serum immunosuppressive cytokines were impacted by immunization. TGF-beta is an immunosuppressive cytokine secreted by tumor stroma and regulatory T cells. We found that the levels of serum TGF-beta decreased significantly in the majority of patients after vaccination. We further analyzed the correlation between the change of serum levels of TGF-beta post vaccination and HER2 ICD vaccine-induced T cell responses. We found that the greater the magnitude of the HER2 specific T cell response, as demonstrated by IFN-gamma secretion, the greater the decrease in serum TGF-beta ($p=0.0045$, $r=0.742$). The correlation between the increased epitope spreading T cell response and decreased levels of TGF-beta was even more significant ($p=0.0003$). The median overall survival has not been reached with 100% of patients alive at this time. Relapse free survival data will be presented.

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